Novel activating and inactivating mutations in the integrin $\beta 1$ subunit A domain

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The ligand-binding activity of integrins is regulated by shape changes that convert these receptors from a resting (or inactive) state to an active state. However, the precise conformational changes that take place in head region of integrins (the site of ligand binding) during activation are not well understood. The portion of the integrin β subunit involved in ligand recognition contains a von Willebrand factor type A domain, which comprises a central β -sheet surrounded by seven α helices ($\alpha 1$ – $\alpha 7$). Using site-directed mutagenesis, we show here that point mutation of hydrophobic residues in the $\alpha 1$ and $\alpha 7$ helices (which would be predicted to increase the mobility of these helices) markedly increases the ligand-binding activity of both integrins $\alpha 5\beta 1$ and

$\alpha 4\beta 1$. In contrast, mutation of a hydrophilic residue near the base of the $\alpha 1$ helix decreases activity and also suppresses exposure of activation epitopes on the underlying hybrid domain. Our results provide new evidence that shifts of the $\alpha 1$ and $\alpha 7$ helices are involved in activation of the A domain. Although these changes are grossly similar to those defined in the A domains found in some integrin α subunits, movement of the $\alpha 1$ helix appears to play a more prominent role in βA domain activation.

Key words: activation, A domain, conformational change, integrin.

INTRODUCTION

Integrins are α, β heterodimeric cell surface receptors that mediate the adhesion of cells to each other and to extracellular matrix macromolecules [1]. The interaction of integrins with their ligands is dependent upon signals transduced from the cytoplasmic tails to the extracellular domains [2]. These signals convert integrins from a constitutive low-affinity state to high-affinity state as a result of conformational changes in the extracellular domains [3,4]. Spatio-temporal regulation of cell adhesion is of critical importance in many fundamental biological processes [5,6].

Different conformational states of integrins can be distinguished by the expression of so-called ‘activation epitopes’: expression of these epitopes is higher on the active or ligand-occupied integrin compared with the inactive integrin. Examples of monoclonal antibodies (mAbs) directed against the $\beta 1$ subunit that recognize activation epitopes are 12G10, 15/7 and HUTS-4 [7–9]. Conversely, expression of the epitopes of some function blocking mAbs is decreased upon activation/ligand recognition [10–12].

The recent crystal structures of the extracellular domains of $\alpha V\beta 3$ [13,14] have provided new insights into integrin activation and ligand recognition. Overall, the integrin structure resembles that of a ‘head’ on two ‘legs’. The head region contains a seven-bladed β -propeller in the α subunit, the upper surface of which is in close juxtaposition with a von Willebrand factor (vWF) type A domain in the β subunit (βA). βA (also referred to as the I-like domain or βI -domain) contains a central β -sheet encircled by seven α helices. The α helices are amphipathic: they have a hydrophobic face that points towards the β -sheet, and a hydrophilic face

that points away from it. βA is connected at its N- and C-termini to an immunoglobulin-like ‘hybrid’ domain and forms an extensive interface with it. The key regions involved in ligand recognition are loops on the upper surface of the β -propeller and the top face of βA , which contains a metal-ion-dependent adhesion site (MIDAS). The interaction of an acidic residue in integrin ligands with a divalent cation at the MIDAS, explains the absolute dependence of integrin-ligand interaction on metal ions [14]. A small number of subtle conformational changes between the unliganded and liganded crystal conformers of $\alpha V\beta 3$ were observed [14], which included an inward shift of the $\alpha 1$ helix in βA .

A sub-family of the integrins also include an A domain in their α subunits (αA); where present, this domain is the major site of ligand binding. αA domains have been crystallised in two forms: ‘closed’ (inactive) and ‘open’ (active) [15]. The major conformational changes involved in activation of these domains include a small inward movement of the $\alpha 1$ helix and a large downward shift of the C-terminal $\alpha 7$ helix [16]. Mutations that perturb the interaction of the $\alpha 7$ helix with the core of the domain have been shown to lead to activation [17–19]. Similarly, locking the position of the $\alpha 7$ helix in either the ‘up’ or ‘down’ position leads to constitutive inactivation or constitutive activation, respectively [20]. Recently we have presented evidence that similar movements of the $\alpha 1$ and $\alpha 7$ helices may take place in the $\beta 1$ subunit A domain [21,22]. For example, changes in the expression of the 12G10 epitope in βA appear to be caused by a movement of the $\alpha 1$ helix [21], whereas a change in the exposure of the 15/7 and HUTS-4 epitopes in the hybrid domain appears to be due to a swing of the hybrid domain away from the α subunit caused by a shift

Abbreviations used: αA , α subunit von Willebrand factor (vWF) A-domain; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); βA , β subunit vWF A-domain (also referred to as the I-like domain or βI -domain); mAb, monoclonal antibody; MIDAS, metal-ion-dependent adhesion site; $\alpha 4\beta 1$ -Fc, C-terminally truncated $\alpha 4$ and $\beta 1$ subunits fused to the Fc region of human IgG; $\alpha 5\beta 1$ -Fc, recombinant soluble integrin heterodimer containing C-terminally truncated $\alpha 5$ and $\beta 1$ subunits ($\alpha 5$ residues 1–613 and $\beta 1$ residues 1–455) fused to the Fc region of human IgG $\gamma 1$; V142A etc., substitution of Val¹⁴² with alanine etc.; VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor.

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of the $\alpha 7$ helix [22]. Furthermore, mutation of a residue at the base of $\alpha 7$ helix of βA (Leu³⁵⁸) resulted in activation of $\alpha 5\beta 1$ [22]. However, some of these conclusions have been challenged; in particular it has been proposed that changes in 12G10 binding could be explained by changes in exposure of the epitope due to activation-induced changes in quaternary structure [23]. The role of $\alpha 7$ helix movement in integrin activation also remains controversial, because no shift of the $\alpha 7$ helix was observed in the ligand-occupied form of $\alpha V\beta 3$ [14]. Furthermore, since Leu³⁵⁸ lies near the interface of βA with the hybrid domain, it is possible that mutation of this residue could cause activation by affecting the interface, rather than by a direct effect on the $\alpha 7$ helix. However, if βA domain activation is similar to that of αA domains, it would be predicted that mutations that alter the mobility of the $\alpha 1$ and $\alpha 7$ helices should cause changes in the activation state of the integrin. Therefore, we have performed alanine-scanning mutagenesis on many of the residues that contribute to these helices. Our data identify novel mutations in both the $\alpha 1$ and $\alpha 7$ helices that modulate the ligand-binding activity of integrins $\alpha 5\beta 1$ and $\alpha 4\beta 1$, and hence confirm the importance of these structural elements in receptor activation.

MATERIALS AND METHODS

mAbs

Rat mAbs 16 and 13 recognizing the human $\alpha 5$ and $\beta 1$ subunits respectively were gifts from Dr K. Yamada (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, U.S.A.). Mouse anti-human $\alpha 5$ mAb P1D6 was a gift from Dr E. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.). Mouse anti-human $\alpha 5$ mAb SNAKA52 and mouse anti-human $\beta 1$ mAbs 12G10 and 8E3 were produced as described previously [24,25]. Mouse anti-human $\beta 1$ mAb TS2/16 was a gift from Dr F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Mouse anti-human $\beta 1$ mAbs JB1A and N29 were gifts from Dr J. Wilkins (University of Manitoba, Winnipeg, Canada). Mouse anti-human mAb 15/7 was a gift from Dr T. Yednock (Elan Pharmaceuticals, South San Francisco, CA, U.S.A.). Mouse anti-human mAbs 4B4 and HUTS-4 were purchased from Beckman Coulter (High Wycombe, U.K.) and Chemicon (Harlow, U.K.) respectively. Mouse anti-human $\alpha 4$ mAb 44H6 was purchased from Serotec (Oxford, U.K.). All mAbs were used as purified IgG or, in some assays, as Fab fragments (prepared as described previously [22]).

Proteins

A recombinant fragment of fibronectin containing type III repeats 6–10 (3Fn6-10) was produced and purified as described previously [11]. 3Fn6-10 was biotinylated as before [11,21] using sulphydryl-LC-NHS biotin (Perbio, Chester, U.K.). A recombinant fragment of vascular cell adhesion molecule (VCAM)-1, containing domains 1 and 2 of VCAM fused C-terminally to the C_H2 and C_H3 domains of murine IgG₁, was a gift from Dr A. Henry (Celltech Chiroscience, Slough, U.K.).

Expression vector construction and mutagenesis

C-terminally truncated human $\alpha 5$ and $\beta 1$ constructs encoding $\alpha 5$ residues 1–613 and β residues 1–455 fused to the hinge regions and C_H2 and C_H3 domains of human IgG γ 1 ($\alpha 5\beta 1$ -Fc): $\alpha 5$ (1-613)-Fc and $\beta 1$ (1-455)-Fc, were generated as described previously [26]. To aid the formation of heterodimers, the C_H3 domain of the $\alpha 5$ construct contained a 'hole' mutation, while the C_H3 domain of the $\beta 1$ constructs carried a 'knob' mutation as des-

cribed previously [26]. Mutations in the $\beta 1$ subunit were carried out using oligonucleotide-directed PCR mutagenesis, as described previously [26] or using the Gene Tailor mutagenesis kit (Invitrogen, Paisley, Scotland, U.K.). Oligonucleotides were purchased from MWG Biotech (Southampton, U.K.). The presence of the mutations (and the lack of any other changes to the wild-type sequence) was verified by DNA sequencing.

Transfection of $\alpha 5\beta 1$ -Fc

Chinese hamster ovary cells L761h variant [26] were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, 2 mM glutamine and 1 % non-essential amino acids (growth medium). Flasks (75 cm²) of sub-confluent CHOL761h cells were transfected with 5 μ g of wild-type or mutant $\beta 1$ (1-455)-Fc, and 5 μ g of $\alpha 5$ (1-613)-Fc DNA using LIPOFECTAMINEPLUS^{TM} reagent (Invitrogen, Paisley, Scotland, U.K.) according to the manufacturer's instructions. After 4 d, culture supernatants were harvested by centrifugation at 1000 g for 5 min. Wild-type or mutant heterodimers were purified using Protein A–Sepharose, essentially as described previously [26].

$\alpha 5\beta 1$ -Fc ligand-binding assays

96-Well plates (Costar 1/2-area EIA/RIA; Corning Science Products, High Wycombe, U.K.) were coated with goat anti-human $\gamma 1$ Fc (Jackson Immunochemicals, Stratech Scientific, Luton, U.K.) at a concentration of 2.6 μ g/ml in Dulbecco's PBS (50 μ l/well) for 16 h. Wells were then blocked for 1–3 h with 200 μ l of 5 % (w/v) BSA, 150 mM NaCl, 0.05 % (w/v) NaN₃, 25 mM Tris/HCl, pH 7.4 (blocking buffer). Wells were then incubated with Protein A-purified heterodimers diluted to approx. 1 μ g/ml with 150 mM NaCl, 25 mM Tris/HCl, pH 7.4, (25 μ l/well) for 1–2 h at 22 °C. Wells were then washed three times with 200 μ l of 150 mM NaCl, 25 mM Tris/HCl, pH 7.4, 1 mM MnCl₂ containing 1 mg/ml BSA (buffer A). Biotinylated 3Fn6-10 (0.1 μ g/ml) in buffer A was added to the plate (50 μ l/well) alone, or in the presence of mAbs N29, TS2/16 or 12G10 (5 μ g/ml). The plate was then incubated at 30 °C for 2 h. Unbound ligand was aspirated and the wells washed three times with buffer A. Bound ligand was quantified by addition of 1:500 dilution of ExtrAvidin peroxidase conjugate (Sigma, Poole, U.K.) in buffer A for 20 min at 22 °C (50 μ l/well). Wells were then washed four times with buffer A, and colour was developed using ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] substrate (50 μ l/well). Background binding to BSA-coated wells was subtracted from all measurements. Measurements obtained were the means $\pm$ S.D. of four replicate wells.

Comparison of epitope expression by wild-type and mutant heterodimers

Plates were coated with anti-human Fc and blocked as described above. The blocking solution was removed and cell culture supernatants were added (25 μ l/well) for 1–2 h. All supernatants were assayed in triplicate, and supernatant from mock-transfected cells was used as a negative control. The plate was washed three times with buffer A (200 μ l/well), and anti- $\alpha 5$ or anti- $\beta 1$ mAbs (5 μ g/ml) were added (50 μ l/well). The plate was incubated for 2 h and then washed three times in buffer A. Peroxidase-conjugated anti-rat or anti-mouse secondary antibodies (1:1000 dilution in buffer A; Jackson Immunochemicals) were added (50 μ l/well) for 30 min, the plate washed four times in buffer A, and colour was developed as above. All steps were performed at 22 °C. Results shown are the means $\pm$ S.D. of three independent experiments.

In each assay involving a comparison between wild-type and mutant $\text{tr}\alpha 5\beta 1$ -Fc proteins, the binding of mAb 8E3 (5 $\mu\text{g}/\text{ml}$) was used to normalize for any differences between the amounts of the different heterodimers bound to the wells [22]. Each experiment shown is representative of at least three independent experiments.

Generation of truncated $\alpha 4$ -Fc construct

A construct containing a C-terminally truncated version of the $\alpha 4$ subunit fused to the Fc region of human IgG was generated by PCR, using a previous truncation of the $\alpha 5$ subunit (at Asp⁶¹³) as a model [26]. An appropriate truncation position, Leu⁶²⁴, was chosen by alignment of the $\alpha 4$ subunit amino acid sequence with that of the $\alpha 5$ subunit using CLUSTALW (<http://www.expasy.ch>). This construct incorporates the $\alpha 4$ subunit β -propeller and thigh domains. A 5' *EcoRV* and 3' *PvuII* restriction site were introduced into the construct, which was then cloned into the pIg vector (a gift from Dr D. Simmons, CelltechChiroscience, Harlow, U.K.). This vector encodes the hinge region and the C_H2 and C_H3 domains of human IgG downstream from the multiple cloning site. By incorporating a splice donor sequence (ACTTACCTGT) in the 3' PCR primer, expression of the construct resulted in production of an in-frame integrin-Fc fusion protein.

Transfection of C-terminally truncated $\alpha 4$ and $\beta 1$ subunits ($\text{tr}\alpha 4\beta 1$ -Fc)

All $\text{tr}\alpha 4\beta 1$ -Fc transfections were carried out in COS-1 cells, using lipofectAMINE 2000^{TM} (Invitrogen), according to the manufacturer's instructions. Briefly, cells were grown to approx. 95 % confluency in 75 cm² tissue culture flasks. LipofectAMINE 2000^{TM} reagent (30 μl) was mixed with 750 μl Optimem^{TM} reduced serum medium (Gibco-BRL). Approx. 12 μg of $\text{tr}\alpha 4$ -Fc and $\text{tr}\beta 1$ -Fc DNA was mixed with 750 μl Optimem^{TM}, and added to the lipofectAMINE 2000^{TM} containing medium made as above. After incubation at 22 °C for 20 min, the DNA-lipid complexes were added to cells in 10 ml Optimem^{TM}, and incubated for 3–4 h. Complete growth medium (10 ml) was added after this period, and this medium replaced by 20 ml complete growth medium 24 h post-transfection. For integrin-Fc production, growth medium was harvested 4 days post-transfection, and used in subsequent experiments.

$\text{Tr}\alpha 4\beta 1$ -Fc ligand-binding assays

96-Well plates were coated with anti-human Fc and blocked as described above. Wells were washed once with 200 μl buffer A, and 185 μl of culture supernatant was added to the wells for 1–2 h, to allow the capture of the $\text{tr}\alpha 4\beta 1$ -Fc proteins. Wells were washed three times with buffer A, and VCAM-mouse Fc (10 $\mu\text{g}/\text{ml}$), diluted in buffer A, added in the absence or presence of anti- $\beta 1$ mAb Fab fragments (5 $\mu\text{g}/\text{ml}$). Plates were incubated at 30 °C for 3 h. Wells were then washed three times, and bound VCAM detected by addition of an anti-mouse Fc-peroxidase conjugated secondary antibody (1:1500 in buffer A) for 45 min. Wells were washed four times, and 50 μl ABTS substrate added. Absorbance readings at 405 nm were measured using a multi-scan plate reader. Background binding (to wells treated with culture supernatant from untransfected cells) was subtracted from all measurements. Measurements obtained were the means $\pm$ S.D. of four replicate wells. Results shown are representative of at least three independent experiments. Binding of the anti- $\alpha 4$ antibody 44H6 was used to normalize for any differences between the amounts of wild-type and mutant proteins captured on the wells.

Table 1 Alanine-scanning mutagenesis of residues within, or adjacent to, the $\alpha 1$ and $\alpha 7$ helices of the $\beta 1$ subunit A domain

Mutations shown in bold caused activation of ligand binding, whilst that underlined caused partial inactivation. The other mutations had little or no effect on ligand binding.

$\alpha 1$ helix region	$\alpha 7$ helix region
L139A	I351A
E140A	D353A
N141A	Y355A
V142A	L358A*
K143A	S359A*
L145A	
T147A	
L149A	
N151A	
M153A	
R154A	
R155A	
I156A	
<u>T157A</u>	
S158A	

* See reference [22].

Homology modelling of the head region of $\beta 1$

A model of the $\beta 1$ A and hybrid domains was built, based on an alignment against the $\alpha V\beta 3$ crystal structure [13], using the same procedures as described previously [21]. Representation of the structure was produced using the PyMol molecular graphics system (<http://www.pymol.org>) [27].

RESULTS

Novel activating (V142A) and inactivating (T157A) mutations in the $\alpha 1$ helix region

To investigate the mechanism of integrin activation we used a model system for the expression of a truncated version of $\alpha 5\beta 1$, which is fused to the Fc region of the heavy chain of IgG $\gamma 1$ [26]; this system allows for rapid screening of mutants. The recombinant integrin has very low constitutive ligand-binding activity but can be made fully active for ligand binding when treated with the anti- $\beta 1$ mAb 12G10 [22,26]. Previous data suggested that a shift in $\alpha 1$ helix of the βA domain takes place during integrin activation [21]. If this paradigm is correct, mutations that alter the mobility of the helix should cause changes in the activation state of the integrin. Therefore, we performed alanine-scanning mutagenesis on many of the residues that contribute to this helix (Table 1). These residues fall into two classes: the hydrophobic residues (the side chains of which face the central hydrophobic β -sheet), and the hydrophilic residues (the side chains of which mainly face outwards). Mutation of the majority of these residues had no effect on the activation state of $\alpha 5\beta 1$ (see Table 1). However, mutation of the hydrophobic residue Val¹⁴² increased the constitutive activity of the $\text{tr}\alpha 5\beta 1$ -Fc (Figure 1). The partial activating effect of this mutation was particularly apparent in combination with the weakly activating mAb TS2/16. Using the more potent activating mAb 12G10, similar levels of ligand binding were observed to the V142A mutant (substitution of Val¹⁴² with alanine) and wild-type receptors. In the same assay the E140A mutation had no effect on the constitutive activity (equivalent to wild-type).

A second mutation, T157A, also affected the activation state but in this case was found to decrease the constitutive activity (Figure 2). This reduced activity was most apparent when the integrin

Figure 1 Effect of $\alpha 1$ helix mutation V142A on binding of 3Fn6-10 fibronectin fragment to $\text{tr}\alpha 5\beta 1\text{-Fc}$

Binding of 3Fn6-10 was measured in the absence of mAbs (open bars) or in the presence of the non function-perturbing mAb N29 (black bars) or the activating mAbs TS2/16 (light-grey bars) or 12G10 (dark-grey bars). The mutation E140A was used as a control. No ligand binding to wild-type or mutant receptors was detected in the presence of 5 mM EDTA (results not shown).

Figure 2 Effect of T157A mutation on binding of 3Fn6-10 fibronectin fragment to $\text{tr}\alpha 5\beta 1\text{-Fc}$

Binding of 3Fn6-10 was measured in the absence of mAbs (open bars) or in the presence of the non-function-perturbing mAb N29 (black bars) or the activating mAbs TS2/16 (light-grey bars) or 12G10 (dark-grey bars). The mutant S158A was used as a control. The T157A mutant has a lower basal level of ligand-binding activity than wild-type or S158A mutant ($P < 0.01$ by Student's *t* test), and a lower level of ligand-binding activity after activation with TS2/16 ($P < 0.01$ by Student's *t* test).

was treated with TS2/16: this mAb increased the activity of the T157A mutant to a much lesser extent than the wild-type receptor or a control mutant S158A. The maximal level of ligand-binding activity achieved in the presence of 12G10 was, however, similar for the T157A mutant and wild-type integrin.

To examine whether the V142A or T157A mutations had any detectable effect on the conformation of the receptor, we used a panel of mAbs to the different domains of the α and β subunits (Table 2). The results showed that the V142A mutation had little effect on the expression of the epitopes recognised by these mAbs,

Table 2 Summary of mAb reactivity with novel $\beta 1A$ domain mutants

Chinese hamster ovary L761h cells were transfected with $\alpha 5(1-613)\text{-Fc}$ and wild-type or mutant $\beta 1(1-455)\text{-Fc}$. Cell culture supernatants were analysed for reactivity with anti- $\alpha 5$ and anti- $\beta 1$ mAbs (used at 5 $\mu\text{g/ml}$) by sandwich ELISA. Assays were performed in the presence of 1 mM Mn^{2+} . +++, reactivity 80–120 % of wild-type integrin; ++, reactivity 50–80 % of wild-type integrin; +, reactivity 20–50 % of wild-type integrin; +/-, reactivity < 20 % of wild-type integrin; N.D., not determined. The E140A mutant was used as a control.

		$\beta 1$ mutant		
mAb	Epitope	V142A	T157A	I351A
$\alpha 5$ subunit β -propeller				
P1D6	W3	+++	+++	++
16	W2/W3	+++	+++	+++
SNAKA52	W2	+++	+++	+++
$\beta 1$ subunit A domain				
13	207–218	++	+++	++
TS2/16	207–218	+++	+++	+++
4B4	207–218	+++	+++	+++
12G10	207–218, 154/155	+++	++	+++
Hybrid domain				
JB1A	82–87	+++	+++	N.D.
15/7	355–425	+++	+/-	+++
HUTS-4	355–425	+++	+/-	+++
PSI domain				
N29	14–55	+++	+++	+++

apart from slightly reduced expression of that of the function-blocking mAb 13. In some assays, enhanced expression of the HUTS-4 and 15/7 activation epitopes was observed for the V142A mutant (approx. 120 % of wild-type levels, results not shown). In contrast, the T157A mutant showed much decreased expression of both the 15/7 and HUTS-4 epitopes (less than 10 % of wild-type levels in the case of the 15/7 epitope). This mutant also showed slightly reduced expression of the 12G10 activation epitope. Therefore, it appears that the T157A mutant causes the integrin to adopt an inactive conformation. This finding is in accord with its low ligand-binding activity.

A novel activating mutation (I351A) in the $\alpha 7$ helix

Previously we have provided evidence for movement of the $\alpha 7$ helix during activation and showed that mutation of Leu³⁵⁸ (at the base of the $\alpha 7$ helix) to alanine causes activation of $\alpha 5\beta 1$ [22]. Using the same rationale as described above for the $\alpha 1$ helix, we tested whether mutation of other residues in the $\alpha 7$ helix could also cause activation (Table 1). The results showed that mutation of the hydrophobic residue Ile³⁵¹ increased the constitutive activity of $\text{tr}\alpha 5\beta 1\text{-Fc}$, but mutation of the other residues had no effect. The activating effect of the I351A mutation was similar to that described above for the V142A mutation: a small increase in the level of constitutive ligand binding was observed, and full activation occurred in the presence of TS2/16 (Figure 3). Similarly to the V142A mutation, the I351A mutation had little effect on the expression of the epitopes recognized by conformation-sensitive mAbs, apart from slightly reduced expression of that of mAb 13 (Table 2).

The V142A, I351A and L358A mutations also cause activation of $\alpha 4\beta 1$

We have shown that mutations in the $\alpha 1$ and $\alpha 7$ helices described above activate $\text{tr}\alpha 5\beta 1\text{-Fc}$, but to test whether these mutations could also activate other $\beta 1$ integrins we examined their effect on

Figure 3 Effect of $\alpha 7$ helix mutation I351A on binding of 3Fn6-10 fibronectin fragment to $\alpha 5\beta 1$ -Fc

Binding of 3Fn6-10 was measured in the absence of mAbs (open bars) or in the presence of the non-function-perturbing mAb N29 (black bars) or the activating mAbs TS2/16 (light-grey bars) or 12G10 (dark-grey bars). The mutant D353A was used as a control. No ligand binding to wild-type or mutant receptors was detected in the presence of 5 mM EDTA (results not shown).

the activity of $\alpha 4\beta 1$. For these experiments we used a truncated form of $\alpha 4\beta 1$ equivalent to that for $\alpha 5\beta 1$ (designated $\text{tr}\alpha 4\beta 1$ -Fc). Similarly to $\text{tr}\alpha 5\beta 1$ -Fc, this construct had very low constitutive ligand-binding activity in the absence of activating mAbs. The results showed that the V142A and I351A mutations, as well as the L358A mutation [22], caused activation of $\text{tr}\alpha 4\beta 1$ -Fc binding to VCAM-1 (Figures 4A and 4B). Control mutations such as E140A or S359A did not cause activation. Hence, these results suggest the mechanism of activation found for $\alpha 5\beta 1$ is applicable to other integrins.

DISCUSSION

We tested the hypothesis that mutations that alter the mobility of the $\alpha 1$ and $\alpha 7$ helices in the A domain of the $\beta 1$ subunit will affect the activation state of $\beta 1$ integrins. Our results show that (i) the mutations V142A (in the $\alpha 1$ helix) and I351A (in the $\alpha 7$ helix) cause partial activation of $\alpha 5\beta 1$ and $\alpha 4\beta 1$, and (ii) the mutation T157A (near the base of the $\alpha 1$ helix) causes partial inactivation. The location of these mutations is shown in Figure 5. It is important to point out that the mutations in the βA domain we describe here are not equivalent to disulphide locking mutations as described in αA domains [20], and would be expected to produce only a partial (rather than a complete) shift of equilibrium to the active or inactive conformation.

The residues that form the α helices in the βA domain fall into two classes: hydrophobic residues (the side chains of which point towards the central hydrophobic β -sheet), and hydrophilic residues (the side chains of which mainly point outwards). Only mutation of the hydrophobic residues, such as Val¹⁴² and Ile³⁵¹, are predicted to increase the mobility of the helices because these mutations should weaken the interaction of the helices with the hydrophobic core of βA . Conversely, Thr¹⁵⁷ lies in the loop at the base of the $\alpha 1$ helix that connects $\alpha 1$ to the following β -strand; substitution of this hydrophilic residue for the more hydrophobic alanine may constrain movement of the $\alpha 1$ helix, because this substitution would increase the interaction of this region with the

Figure 4 Effect of $\alpha 1$ and $\alpha 7$ helix mutations on VCAM binding to $\text{tr}\alpha 4\beta 1$ -Fc

(A) V142A mutation in the $\alpha 1$ helix, (B) I351A and L358A mutations in the $\alpha 7$ helix. Binding of a recombinant fragment of VCAM-1 (containing domains 1 and 2) fused to Fc region of mouse IgG was measured in the absence of mAbs (open bars) or in the presence of Fab fragments of the non function-perturbing mAb N29 (black bars) or the activating mAbs TS2/16 (light-grey bars) or 12G10 (dark-grey bars). The mutants E140A and S359A were used as controls in panels (A) and (B) respectively. No ligand binding to wild-type or mutant receptors was detected in the presence of 5 mM EDTA (results not shown).

core of the domain. Significantly, the residue at this position in all β subunits is invariably a hydrophilic residue (Thr/Ser/Gln).

A 'gain-of-function' mutation in the $\alpha 1$ helix region of the $\beta 3$ subunit has been reported previously [28]. In this mutation Trp¹²⁹-Asn¹³³ of $\beta 3$ was swapped with the equivalent residues from $\beta 1$ (Glu¹⁴⁰-Ser¹⁴⁴) and resulted in activation of $\alpha \text{IIb}\beta 3$. Importantly this swapping mutation exchanged Ile¹³¹ of $\beta 3$ with the less hydrophobic valine, and it is likely that this exchange contributed to the activating effect of the mutation (note that Ile¹³³ in $\beta 3$ is equivalent to Val¹⁴² in $\beta 1$).

Expression of the 15/7 and HUTS-4 epitopes in the hybrid domain was only very slightly increased by the V142A and I351A mutations, in contrast with marked increase in expression of these epitopes observed for the L358A mutation [22]. A possible explanation is that the V142A and I351A mutations are less strongly activating than L358A, and result in an intermediate

Figure 5 Homology model of the $\beta 1$ subunit A and hybrid domains showing the location of activating and inactivating mutations

The side chains of residues Val¹⁴², Ile³⁵¹ and Leu³⁵⁸, (which cause activation when mutated) are shown in green; the side chain of residue Thr¹⁵⁷ (which causes inactivation when mutated) is shown in red. For the A domain, α helices are shown in orange and β strands in blue. The divalent ion at the MIDAS site is shown as a purple sphere. The hybrid domain (shown only in part) is depicted in grey.

state of activation in which there is little or no movement of the hybrid domain. Such an intermediate state of activation is observed in αA domains, in which there is only a small downward shift of the $\alpha 7$ helix [29]. However, decreased binding of mAb 13 (the epitope of which is attenuated in active/ligand-occupied state) was observed for the V142A and I351A mutants, as was seen for the L358A mutant [22], suggesting that the mutations do cause conformational changes within βA . Altered binding of this mAb appears to be due to conformational changes in the $\alpha 2$ helix region, where its epitope is located [14,30]. The T157A mutation caused almost complete loss of 15/7 and HUTS-4 binding. This loss of binding is not due to a direct effect on the 15/7 and HUTS-4 epitopes, since these epitopes lie entirely within the hybrid domain and are spatially distant from Thr¹⁵⁷ [22]. Instead, these data indicate that movement of the hybrid domain is compromised [22]. Since Thr¹⁵⁷ lies at the interface of βA with the hybrid domain this effect on hybrid domain movement could be due to a perturbation of the interface. However, we consider it more likely that the T157A mutation affects movement of the $\alpha 1$ helix because (i) mutation of Asp¹³⁷ or Asp¹³⁸ at the top of the $\alpha 1$ helix causes similar attenuation of the 15/7 and HUTS-4 epitopes [31], (ii) decreased expression of these epitopes is also caused by function-blocking mAbs which bind of the side of the A

domain in the $\alpha 1/\alpha 2$ helix region [22], and (iii) mutation of Ser¹⁵⁸ (also at the interface) did not affect the activation state or exposure of 15/7 and HUTS-4 epitopes. In further support of a direct effect of the T157A mutation on the movement of the $\alpha 1$ helix, is the observation that the mutation caused decreased expression of the 12G10 epitope; this epitope includes residues in the $\alpha 1$ helix [21]. Somewhat surprisingly, the binding of 12G10 to the T157A mutant overcame the effect of the mutation, perhaps because this 'forced' the $\alpha 1$ helix to adopt the active ('in') position [21,22].

Mutation of Val¹⁴² or Ile³⁵¹ had very similar consequences, even though these residues lie in different helices and are widely separated in the tertiary structure of βA . This finding suggests that movement of the $\alpha 1$ helix or the $\alpha 7$ helix produces a similar effect on the overall conformation of βA . As shown previously, movement of the $\alpha 1$ and $\alpha 7$ helices in the βA domain suggests that they appear to be linked in a similar manner as in αA domains [22]. It is not immediately clear why mutation of only a small subset of the hydrophobic residues in the $\alpha 1$ and $\alpha 7$ helices results in activation. However, due to the pitch of the α helix, some of the hydrophobic side chains point directly towards the β -sheet whereas others are more offset. Therefore, some hydrophobic residues may make a large contribution to the free energy of the interaction of the α -helix with the β -sheet, whereas others may contribute little. Significantly, the Val¹⁴², Ile³⁵¹ and Leu³⁵⁸ side chains point directly towards the hydrophobic core (Figure 5).

Mutations in αA domains that cause activation of ligand-binding cluster around the $\alpha 7$ helix, and are proposed to perturb the interaction of $\alpha 7$ with the body of the domain [17–19,32]. Specifically, $\alpha 7$ has been shown to interact with a hydrophobic pocket into which fits a conserved isoleucine residue at the base of $\alpha 7$ [18]. We have now described similar mutations in βA , although we have not found any evidence in support of an identical hydrophobic pocket [22]. In contrast with the βA domain, no activating $\alpha 1$ helix mutations have been described in αA domains. Furthermore, unlike for the βA domain [21,30,33–35], no activating or allosteric function-blocking mAbs that bind in the $\alpha 1$ helix region have been reported for αA domains. Taken together, these findings suggest that $\alpha 1$ helix movement may play a more prominent role in βA domain activation than it does in αA domain activation.

A movement of the $\alpha 1$ helix, but not of the $\alpha 7$ helix, is observed in the 'liganded' state of $\alpha V\beta 3$ (although the preceding $\beta 6$ - $\alpha 7$ loop undergoes a large shift [14]). As proposed previously [16,23] this lack of $\alpha 7$ movement may be due to the bent state of the integrin in the crystal lattice, which restrains any movement of the hybrid domain. Although this receptor can bind a cyclic RGD ligand, it has been shown that small RGD-containing peptides can bind to integrins in an activation-independent manner [36]. Hence, it is likely that the structure of the RGD-bound receptor [14] corresponds to a low affinity state.

In summary, our results support the hypothesis that, upon activation, the βA domain undergoes conformational changes involving shifts in the $\alpha 1$ and $\alpha 7$ helices. Disregulated activation of integrins may play a role in human diseases such as Glanzmann's thrombasthenia and cancer [37,38]. Our findings suggest that it may be possible to design novel allosteric inhibitors of βA domain activation, similar to the allosteric inhibitors of αA domains [39], for therapeutic use.

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